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Poxvirus Proteomics and Virus-Host Protein Interactions

Kim Van Vliet,† Mohamed R. Mohamed,† Leiliang Zhang,† Nancy Yaneth Villa, Steven J. Werden, Jia Liu, and Grant McFadden*

Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, Gainesville, Florida

INTRODUCTION

Poxviruses are large, enveloped, double-stranded DNA viruses, the majority of which possess over 200 genes. Large-scale DNA sequencing projects have provided a wealth of information regarding poxvirus genomes (www.poxvirus.org). However, the function by which the majority of these genes contribute to poxvirus biology remains poorly understood. Comparative DNA sequencing studies have revealed that genes centrally located in the poxvirus genome tend to be more highly conserved among poxvirus family members and play roles in fundamental biological processes such as virus assembly and replication. In contrast, viral genes that tend to cluster near the ends of the genome, either within the terminal inverted repeats or near terminal regions, are much more diverse and encode unique biological determinants such as host range and virulence factors or play a role in enabling the virus to evade recognition and clearance by the host immune system (142). As the sequence databases of large virus genomes continue to grow, it has become increasingly important to understand the expression, function, and regulation of the entire proteome encoded by their genomes. In this review, we focus on recent advances in poxvirus proteomic studies, including those analyzing the protein composition of the poxvirus virion, viral protein-viral protein interactions, global poxviral protein expression studies, structural biology of poxvirus proteins, as well as yeast two-hybrid (Y2H) studies aimed at identifying virus-host interactions. In addition, we discuss a few relevant technologies that have recently been developed for high-throughput studies of protein-protein interactions (PPIs).

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, 1600 SW Archer Road, Box 100266, Gainesville, FL 32610. Phone: (352) 273-6852. Fax: (352) 273-8905. E-mail: grantmcf@ufl.edu.

† Kim Van Vliet, Mohamed R. Mohamed, and Leiliang Zhang contributed equally to this work.

PROTEOMIC STUDIES OF POXVIRUS VIRIONS

Knowledge of the protein composition of the infectious viral particle, or virion, is an important prerequisite for functional studies, but obtaining this information for larger virions with more complex structures can be challenging. With dimensions of 360 by 270 by 250 nm (34), poxviruses are among the largest and most complex of all animal viruses. Poxviruses express three temporal classes of genes, denoted early, intermediate, and late, and some authors have suggested that the early class of genes can be further subdivided into early and immediate-early genes (6). The late genes encode the lion's share of the virion structural proteins and morphogenesis factors required for the assembly of new virus particles, whereas many of the enzymes packaged in the virion core can be encoded by early or early/late genes (reviewed in reference 110). Mature poxvirus virions lack the symmetry features common to other viruses, such as an obvious helical or icosahedral capsid architecture; however, studies have shown that early in the assembly of vaccinia virus (VACV), spherical immature virions are produced from trimers of the D13 protein, which forms a honeycomb lattice structure, suggesting that the infectious form of the ancestor of poxviruses may have had an icosahedral capsid (14, 162). Following morphogenesis, mature poxvirus virions appear as brick-shaped membrane-bound particles with a complex internal structure featuring a walled, biconcave core that is flanked by two lateral bodies (31). There are four infectious forms of poxvirus virions, the intracellular mature virus (IMV), the intracellular enveloped virus, the cell-associated extracellular enveloped virus, and the extracellular enveloped virus, all of which share the same IMV at their center, which contains one membrane, a concave brick core, and protein-based lateral bodies. Recently, an alternative nomenclature was proposed, designating the IMV as mature virions (MVs), designating the intracellular enveloped virus as wrapped virions (WVs), and referring to both the cell-associated extracellular enveloped virus and the extracellular enveloped virus as extracellular virions (EVs) (111). Throughout this review, the most recent nomenclature designations will be used.

Although the complete genomic sequences of most poxvi-

ruses have been available for many years (see www.poxvirus.org), only a few studies have attempted a comprehensive survey of the protein composition of poxvirus virions. As the prototypical and best-characterized member of the poxvirus family, VACV was the focus of most of these attempts. Early studies attempted to analyze the protein composition of purified VACV MVs utilizing mainly sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under different electrophoretic conditions (54, 100, 120, 121, 140). However, the early nomenclature for the proteins identified in these studies was based on their electrophoretic migration on SDS-PAGE gels, which made it difficult to compare results between different studies. Later, a combination of SDS-PAGE and N-terminal amino acid sequencing was employed to identify 12 unique virus-encoded proteins from purified MVs of the IHD-J strain of VACV (163) (Table 1). Likewise, through the use of two-dimensional gel electrophoresis followed by either matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) or N-terminal amino acid sequencing (Edman degradation) or by using immunoprecipitation with defined antibodies, 12 unique major membrane and core proteins from MVs of the Western Reserve (WR) strain of VACV (VACV-WR) were identified (78) (Table 1). However, most of these studies were limited in their ability to perform a complete structural analysis of all the protein components of the virion, possibly due to the limited availability of advanced purification techniques and the limitations of the technologies available for protein identification at the time of those studies.

Ten years later, several studies of the comprehensive structural analysis of the protein composition of the infectious MV of VACV were reported (30, 134, 179). In one study, by analyzing the proteins in the MV of VACV-WR using a combination of gel-free liquid chromatography and tandem MS (LC-MS/MS), Chung et al. identified 75 viral proteins and determined their relative abundances (Table 1) (30). The majority of the viral proteins identified from MV particles were consistent with prior reports and included enzymes, transcription factors, membrane proteins, core proteins, and host-interacting proteins. Notably, 10 previously unknown components of the MV were identified (Table 1). Analysis of the relative abundances of the identified viral proteins revealed that A4 (43, 78) was the most abundant protein in the MV particles (Table 1).

In a similar study published that same year, Yoder et al. reported the results of a comprehensive proteomic study of the protein composition of the VACV virion (Copenhagen strain [Cop]) (179). In their analysis, the authors utilized either reverse-phase high-performance liquid chromatography or SDS-PAGE in combination with either a MALDI-time of flight (TOF) tandem mass spectrometer (MALDI-TOF/TOF), an LC electrospray ionization quadrupole ion trap mass spectrometer, or an LC electrospray ionization quadrupole-TOF mass spectrometer. Although not quantitative, their analysis identified 63 VACV virion proteins and confirmed the presence of most previously known VACV virion proteins (Table 1). However, some of the VACV proteins previously identified by Chung et al. (30) were not identified in that study (A2.5, A6, A9, A18, A21, A22, A25, A26, A28, A31, A45, C6, D7, D13, G5.5, G9, H2, H6, I2, and L5), and the data set also included a number of proteins, mostly EV proteins (such as F13, A56, B5, A33, and A34) that were not identified by Chung et al. (30), possibly as a result of the method used to purify MV particles, which may have selectively stripped some EV proteins from the viral particles (Table 1). Finally, one more comprehensive analysis of the protein composition of purified MV particles of VACV-WR was reported by Resch et al. (134). For their analysis, Resch et al. used MV particles that were purified by using two successive rate-zonal centrifugations in sucrose gradients followed by isopycnic banding in cesium chloride (134). Their analysis identified a total of 80 virion proteins, including 69 previously reported virion components and 11 that were not previously reported to be components of virions (134). However, 15 previously described virion proteins were not identified in their analysis, possibly due to technical reasons including small size and hydrophobicity. Based on their rough calculations of protein abundance and molecular weight, Resch et al. (134) determined the 10 most abundant MV proteins, which included the known major MV proteins F17, A3, A4, A10, and A17, and concluded that they account for approximately 80% of the MV protein mass, in agreement with data reported previously (30, 140).

Interestingly, none of the three previous comprehensive MS studies discussed above (30, 134, 179) identified protein products from any novel viral open reading frames (ORFs) that had not been annotated in the published VACV-WR genome sequence (GenBank accession number NC_006998). Additionally, one particular VACV-Cop virion core protein (originally designated ORF F18R but now denoted F17R) identified previously by Jensen et al. (78) was not identified in either of these comprehensive analyses. Consolidating data from all reported proteomic studies of VACV MVs, a consensus MV complement of 73 proteins was identified by at least two studies. An additional 22 proteins were identified as being virion components by only one study, bringing the total number of reported VACV MV proteins to 95, which is very close to earlier estimates that the VACV virion contains around 100 proteins (54). However, two proteins, A14.5 (12) and G5 (35), were previously described to be MV proteins but were not detected in any of the above-mentioned studies and therefore were not included in Table 1. These studies highlight the important role that different proteomic technologies have played in deciphering the components of the complex poxvirus virion as well as the importance of defining virion purity with precision.

Due to renewed interest in studies of poxvirus pathogenesis, the proteomes of two orthopoxviruses with differing pathogenic potentials, monkeypox virus (MPXV) and VACV, were compared with the goal of identifying proteins that might influence pathogenesis (98). In that study, both MV and EV infectious particles from MPXV and VACV were produced in HeLa cells and purified using sucrose gradient ultracentrifugation. These virions were determined to be >98% pure. MVs and EVs were analyzed by LC-MS/MS, in contrast to previous studies that analyzed only VACV MVs (30, 78, 134, 163, 179), resulting in the identification of a total of 164 viral proteins, from both MPXV and VACV, as being virion associated (i.e., proteins bound to or contained within the virion) (98). Due to a larger number of samples for MPXV (seven MPXV samples versus three VACV samples), more MPXV peptides were identified than VACV peptides. Nevertheless, a comparison of the VACV proteins identified (98) to those detected by the

TABLE 1. VACV MV proteins[a]

VACV-WR ORF	VACV-Cop ORF	Protein function and/or location	Relative abundance[b]		Reference(s)
			Molar %	Wt %	
121	A2.5L	S-S-bond formation pathway; links SH-oxidase E10R and thioredoxin G4L; morphogenesis	0.30	0.10	30, 134
122	A3L	Major core protein; p4b precursor of core protein 4b; morphogenesis	1.98	5.55	30, 134, 163, 179
123	A4L	p39; core protein complexes with core protein p4a/4a; morphogenesis	20.10	24.04	30, 78, 134, 179
124	A5R	DNA-dependent RNA polymerase subunit rpo19	0.85	0.62	30, 134, 179
125	A6L	Unknown; interacts with A21	0.09	0.15	30, 134
126	A7L	Large subunit of early gene transcription factor VETF	0.20	0.65	30, 134, 179
128	A9L	MV membrane protein required for morphogenesis	0.11	0.05	30, 134
129	A10L	Major core protein; C terminus of core protein 4a (p23, amino acids 698-891); complexes with A4L; morphogenesis	1.91	7.54	30, 78, 134, 163, 179
130	A11R	Morphogenesis, viral membrane formation	ND	ND	134
131	A12L	Viral structural protein	ND	ND	134, 163, 179
132	A13L	p8; MV membrane phosphoprotein, morphogenesis	5.76	1.71	30, 78, 134, 163, 179
133	A14L	p16; MV membrane phosphoprotein required for morphogenesis	8.31	3.21	30, 78, 134, 163, 179
135	A15L	Core-associated protein; morphogenesis	0.49	0.21	30, 134, 179
136	A16L	Myristoyl protein; entry/fusion	0.08	0.13	30, 179
137	A17L	MV membrane protein undergoes phosphorylation and proteolytic processing; required for morphogenesis	0.29	0.26	30, 78, 134, 163, 179
138	A18R	DNA helicase affects elongation and termination of postreplicative viral transcription	0.05	0.10	30, 134
139	A19L	Unknown	ND	ND	134
140	A21L	MV membrane protein required for membrane fusion	0.08	0.04	30, 134
142	A22R	Palmityl protein; Holliday junction endonuclease; resolves viral DNA concatemers into unit-length genomes	0.06	0.05	30
144	A24R	DNA-dependent RNA polymerase subunit rpo132	0.25	1.27	30, 134, 179
148	A25L	Gene fragment; CPXV ATI protein	1.04	3.40	30, 134
149	A26L	p4c protein; MV membrane protein required for directing MV into ATI body	1.26	2.82	30, 134
150	A27L	MV membrane protein with roles in MV-cell attachment, fusion, and microtubule transport	8.41	4.09	30, 78, 134, 163, 179
151	A28L	MV membrane protein required for membrane fusion	0.51	0.32	30, 134
152	A29L	DNA-dependent RNA polymerase subunit rpo35	0.15	0.21	30, 134, 179
153	A30L	MV protein for association of dense viroplasm with viral membranes during morphogenesis	0.93	0.31	30, 134, 179
154	A31R	Unknown	ND	ND	30
156	A33R	EV glycoprotein	ND	ND	179
157	A34R	EV glycoprotein	ND	ND	179
167	A42R	Profilin homolog	1.45	0.84	30, 134, 179
171	A45R	Inactive Cu-Zn superoxide dismutase-like in virion	0.30	0.16	30, 134
172	A46R	TIR-like suppresses TIR-dependent signal transduction host defense modulator	0.04	0.04	30, 179
176	A50R	DNA ligase	ND	ND	134
181	A56R	EV glycoprotein, hemagglutinin	ND	ND	179
183	B1R	Ser/Thr kinase essential for viral DNA replication	ND	ND	134
187	B5R	EV membrane glycoprotein	ND	ND	179
196	B22R	Serpin (C16L)	ND	ND	179
022	C6L	Unknown	ND	0.04	30
004	C22L	ITR gene fragment, TNF-α receptor-like	ND	ND	134
106	D1R	Large subunit of mRNA capping enzyme; transcription termination factor VTF; required for intermediate transcription	0.28	1.05	30, 134, 179
107	D2L	Virion core component; morphogenesis	0.54	0.35	30, 134, 179
108	D3R	Virion core component; morphogenesis	0.57	0.61	30, 134, 179
111	D6R	Small subunit of early gene transcription factor VETF; ATPase	0.12	0.34	30, 134, 179
112	D7R	DNA-dependent RNA polymerase subunit rpo18	0.14	0.10	30
113	D8L	p32; MV membrane protein binds cell surface chondroitin and may affect viral entry	1.74	2.39	30, 78, 134, 163, 179
116	D11L	DNA-dependent ATPase (NPHI); transcription elongation, termination, release factor; interacts with H4	0.93	2.59	30, 134, 179
117	D12L	Small subunit of mRNA capping enzyme; transcription termination factor (VTF), intermediate gene transcription	0.50	0.64	30, 134, 179
118	D13L	Rifampin target; scaffold for assembly of the IV membrane	0.02	0.05	30, 134
057	E1L	Poly(A) polymerase catalytic subunit VP55	0.10	0.22	30, 134, 179
059	E3L	dsRNA binding protein; inhibits IFN antiviral activities	ND	ND	134, 179
060	E4L	DNA-dependent RNA polymerase subunit rpo30; intermediate gene transcription factor VITF-1; TFIIS-like	0.21	0.24	30, 134, 179
062	E6R	Unknown	0.68	1.77	30, 134, 179
064	E8R	Core protein implicated in virus assembly and transcription; F10L kinase substrate	0.59	0.73	30, 134, 179
065	E9L	DNA polymerase	ND	ND	134
066	E10R	S-S-bond formation pathway; sulfhydryl oxidase; substrates L1R/F9L; morphogenesis	0.25	0.11	30, 134, 179
067	E11L	Virion core protein; required for MV infectivity	0.08	0.05	30, 134, 179
043	F4L	Ribonucleotide reductase small subunit	ND	ND	134
047	F8L	Protein with iActA-like proline repeats not required for actin tail formation	0.30	0.09	30, 134, 179
048	F9L	Membrane protein; S-S-bond formation pathway; thiol substrate	0.48	0.44	30, 134, 179
049	F10L	Ser/Thr protein kinase; phosphorylates A14 and A17; morphogenesis	0.15	0.31	30, 134, 179
052	F13L	EV membrane protein	ND	ND	134, 179
056	F17R	DNA binding phosphoprotein in virus core; p11 morphogenesis	16.97	7.44	30, 78, 134, 179
078	G1L	Insulin metalloproteinase-like	0.28	0.75	30, 134, 179
079	G3L	Entry/fusion complex	0.42	0.21	30, 179
081	G4L	Glutaredoxin; S-S-bond formation pathway; thioredoxin-like; morphogenesis	0.23	0.12	30, 78, 134, 179
083	G5.5R	DNA-dependent RNA polymerase subunit rpo7	1.13	0.32	30, 134
085	G7L	Virion structural protein; morphogenesis	0.62	1.01	30, 134, 163, 179
087	G9R	Myristyl protein; MV entry/fusion protein complex	0.06	0.08	30, 134
099	H1L	Tyr/Ser protein phosphatase, required for early transcription	0.79	0.60	30, 134, 179
100	H2R	MV membrane protein required for membrane fusion	0.09	0.08	30, 134

Continued on following page

TABLE 1—*Continued*

VACV-WR ORF	VACV-Cop ORF	Protein function and/or location	Relative abundance[b]		Reference(s)
			Molar %	Wt %	
101	H3L	Immunodominant; MV heparin binding surface protein involved in MV maturation	2.13	3.08	30, 78, 134, 163, 179
102	H4L	RNA polymerase-associated protein RAP94; early gene transcription; preinitiation and termination	0.16	0.56	30, 134, 179
103	H5R	Multifunctional; substrate of B1 kinase; roles in DNA replication, transcription, RNA processing, and morphogenesis	0.59	0.50	30, 134, 179
104	H6R	Topoisomerase type IB; important for early transcription	0.26	0.37	30, 134
070	I1L	Encapsidated DNA binding protein required late in infection; morphogenesis	0.12	0.17	30, 134, 179
071	I2L	MV membrane protein required for membrane fusion	2.21	0.73	30
072	I3L	ssDNA binding phosphoprotein	0.16	0.18	30, 134, 179
074	I5L	Minor membrane component of the virion	0.28	0.10	30, 163, 179
075	I6L	Telomere binding protein; morphogenesis	ND	ND	134
076	I7L	Core cysteine proteinase; morphogenesis	0.26	0.50	30, 134, 179
077	I8R	NPHII; RNA/DNA-dependent NTPase; RNA helicase activity	0.22	0.66	30, 134, 179
093	J1R	Virion protein required for morphogenesis	0.82	0.57	30, 134, 179
095	J3R	Stimulatory poly(A) polymerase subunit; cap methyltransferase and transcription elongation factor	0.39	0.59	30, 134, 179
096	J4R	DNA-dependent RNA polymerase subunit rpo22	0.25	0.20	30, 134, 179
097	J5L	Member of MV entry/fusion protein complex	ND	ND	134
098	J6R	DNA-dependent RNA polymerase subunit rpo147	0.25	1.45	30, 134, 179
035	K4L	Homolog to VP37; phospholipase D-like; nicking/joining activity	0.11	0.21	30, 134, 179
088	L1R	M25, MV membrane protein; S-S-bond formation pathway thiol substrate; myristyl protein; morphogenesis	0.14	0.15	30, 78, 134, 179
090	L3L	Required for early transcription by cores	0.16	0.26	30, 134, 179
091	L4R	Major core protein; microtubule-associated ssDNA/ssRNA binding protein	3.20	3.52	30, 78, 134, 163, 179
092	L5R	MV membrane protein required for membrane fusion	0.10	0.06	30, 134
030	M1L	Ankyrin-like	ND	ND	134
028	N1L	Virokine; host defense modulator	ND	ND	134
069	O2L	Nonessential glutaredoxin	0.79	0.38	30, 134, 179

[a] ssRNA, single-stranded RNA; ssDNA, single-stranded DNA; ND, not determined; VETF, vaccinia early transcription factor; VTF, vaccinia termination factor; TIR, Toll/IL-1 receptor; ITR, inverted terminal repeat; TFIIS, transcription factor IIS; iActA, *Listeria ivanovii* protein involved in actin tail formation.
[b] Relative abundance was determined in a study by Chung et al. (30).

three prior comprehensive proteomic investigations of VACV discussed above (30, 134, 179) revealed that only a single VACV protein, I5, was not detected (Table 1). Notably, while the MPXV and VACV proteomes overlapped significantly, eight unique MPXV-specific proteins were identified by LC-MS/MS, including four that are fragmented in VACV (MPXV ORFs 002, 003, 010, and 165), and 22 unique VACV-specific proteins were identified. The unique MPXV-specific virion proteins consisted of three structural proteins and five proteins with previously unknown functions. Interestingly, MPXV ORF 003 encodes an ankyrin (ANK)-containing protein (MPXV-003) that was recently shown to interact with cellular NF-κB1 and Skp1 and was capable of inhibiting NF-κB signaling activity in human cells by stabilizing NF-κB1 and preventing its degradation (108). That study provides a better understanding of the PPIs of an MPXV protein whose function was previously unknown. Such studies of MPXV proteins may be useful for understanding the more severe pathology of MPXV infection in humans and primates than VACV.

Proteomic comparisons of the different forms of infectious MPXV and VACV revealed that EV samples contained significantly different viral protein compositions (48 common to the majority of samples) compared with the MV samples (85 common to the majority of samples) (98). This is in agreement with previous results concluding that the intracellular mechanisms that produce EV from MV significantly alter the viral protein composition of these particles (155). However, it is also possible that since MVs were purified from host cell lysates, these MVs were contaminated with intracellular viral proteins that are not really MV associated, giving a larger number of proteins detected in the MV samples. The EV samples had lower viral titers than MV samples, and it is possible that fewer proteins were detected as a result. Posttranslational modifications of some proteins in the EV samples may have prevented their detection by MS, and this also might have contributed to fewer EV proteins being detected (98). For proteomic identification studies such as these, virion purity is always a critical issue. For example, the D13 protein of VACV functions during the morphogenesis of the immature virion (IV) form, but the protein is normally shed when IVs mature to the MV, and thus, viral proteins like D13 can become remnant "hitchhikers" in MV preparations and may not be actual components of the MV itself (57). In addition to the viral proteins identified by Manes et al. (98), 2,975 host proteins were also identified. Due to the high sensitivy of the MS techniques used, the majority of these proteins are probably contaminants in the virus samples analyzed, such as keratin, heat shock proteins, histone proteins, and ribosomal proteins. However, several host cell proteins such as actin, cofilin, laminin, prohibitin, transgelin, tubulin, and vimentin were identified as being putative virion-associated proteins. MS provides a powerful method for evaluating complex mixtures of proteins of different purities.

In contrast to VACV, little effort has been devoted to proteomic analysis of infectious MV particles from other chordopoxviruses; however, recently, the protein composition of MV particles of the Lausanne strain (Lau) of myxoma virus (MYXV) (MYXV-Lau), a member of the genus *Leporipoxvirus*, was determined (180). Using both reverse-phase high-performance LC and SDS-PAGE in combination with MALDI-TOF MS, 17 different MYXV-Lau capsid proteins

TABLE 2. MYXV MV proteins identified previously[a]

Gene	MYXV-Lau ORF	Description and protein function	VACV-Cop ORF
MYXV-Lau-031	M026R	DNA binding phosphoprotein in virus core; morphogenesis	F17R
MYXV-Lau-048	M043L	Core cysteine proteinase; morphogenesis	I7L
MYXV-Lau-063	M058R	Major core protein; core package/transcription	L4R
MYXV-Lau-065	M060R	Virion protein required for morphogenesis	J1R
MYXV-Lau-073	M068R	DNA-dependent RNA polymerase subunit rpol47	J6R
MYXV-Lau-076	M071L	MV heparin binding surface protein involved in MV maturation	H3L
MYXV-Lau-077	M072L	RNA polymerase-associated protein RAP94; early gene transcription, preinitiation, and termination	H4L
MYXV-Lau-091	M086L	DNA-dependent ATPase (NPHI)	D11L
MYXV-Lau-097	M092L	Major core protein; p4b (processed); morphogenesis	A3L
MYXV-Lau-098	M093L	Core protein	A4L
MYXV-Lau-100	M095L	Virion morphogenesis protein	A6L
MYXV-Lau-101	M096L	Large subunit of early gene transcription factor VETF	A7L
MYXV-Lau-104	M099L	Major core protein p4a (processed); morphogenesis	A10L
MYXV-Lau-117	M112R	Holliday junction endonuclease; resolves viral DNA concatemers into unit-length genomes	A22R
MYXV-Lau-120	M115L	MV membrane protein with roles in MV-cell attachment, fusion, and microtubule transport	A27L
MYXV-Lau-136	M131R	Cu-Zn superoxide dismutase-like in virion	A45R
MYXV-Lau-155	M151R	Serpin ("Serp2")	B13R

[a] Based on data from reference 180. VETF, vaccinia early transcription factor.

were identified (Table 2). Most of the MYXV proteins identified in that study were orthologs of either VACV structural proteins or virion-associated enzymes, suggesting that leporipoxviruses utilize the same conserved core assembly and protein cleavage pathways employed by orthopoxviruses (180). One difference was that M151R, an ortholog of the VACV B13R protein that was not previously known to be a capsid component, was also detected in MYXV core fractions. Moreover, M093L, a protein of unknown function that lacks any known orthopoxvirus ortholog, was shown to associate with membrane fractions (180). In conclusion, it is clear that these comprehensive proteomic analyses of poxvirus virions have enabled the identification of many virion components, some of which are likely to be involved in the earliest stages of infection and pathogenesis. The identification of these components is just a starting point for further studies to elucidate the functional role that each factor plays in poxvirus biology.

VIRUS-VIRUS PROTEIN INTERACTIONS

During the life cycle of large complex viruses, there are extensive PPIs among the virus-encoded proteins. Many viral proteins assemble as a functional group, such as the VACV entry-fusion complex (EFC) (111). These VACV PPIs have been systematically studied by Y2H screening. In that study, each of the 226 predicted ORFs were expressed in *Saccharomyces cerevisiae* and evaluated for pairwise PPIs, and out of nearly 70,000 combinations tested, 37 PPIs were specifically identified (101). Some of these PPIs shed light on the functional relevance of previously uncharacterized proteins; for example, the A2.5 protein was shown to interact with the product of the E10R gene (149). On the other hand, there also remains a large number of previously described viral PPIs that simply do not register as positive hits by the Y2H assay method (31). Efforts to express some proteins in yeast, for example, VACV protein E9, have been unsuccessful; therefore, these PPIs, although detectable using other means, were not detected in Y2H assays. Additionally, codon optimization may be needed to improve the levels of VACV protein expression in yeast in order to detect them. Regardless, the functional characterization of individual poxvirus proteins has produced large amounts of new data regarding viral PPIs, which will prove vital to an understanding of the role that viral proteins play in various biochemical pathways. In this section, we summarize important PPIs as they pertain to known biochemical pathways (Table 3) and then discuss various viral PPIs that are thought to be important during different stages of the poxvirus life cycle.

Poxviruses encode many enzymes that catalyze a variety of macromolecular reactions in different biochemical pathways. First, we focus on the protein interactions between viral enzymes and viral substrates. Infectious MVs are assembled in the cytoplasm and subsequently wrapped with an additional membrane derived from the *trans*-Golgi or endosomal cisternae before exocytosis to become EV particles. Some MV proteins possess stable disulfide bonds in their cytoplasmic domains, which could not have been acquired through known cellular pathways that normally would occur in the oxidizing environment of the lumen of the endoplasmic reticulum (147). A number of proteins, including not only viral core proteins but also the cytoplasmic surface of some viral integral membrane proteins, which do not reside in the lumen of the endoplasmic reticulum, become disulfide bonded during assembly (97). To allow for the formation of stable disulfide bonds in this cytoplasmic compartment, poxviruses exploit a novel virus-specified cytoplasmic redox pathway (147, 148). Studies of this pathway revealed that the key virus-encoded components can be divided into two groups, viral redox-active proteins such as E10, A2.5, and G4 (146, 149, 174) and viral substrate proteins such as A16, A21, A28, F9, G9, H2, J5, L1, and L5 (13, 122, 145, 164, 165) (Table 3). Three viral redox-active proteins (E10, A2.5, and G4) play a role in the cytoplasmic redox

TABLE 3. PPIs between VACV proteins that regulate biochemical pathways

Pathway	VACV proteins	Function
Poxvirus cytoplasmic redox pathway	Viral redox-active proteins (E10, A2.5, and G4) Viral substrate proteins (A16, A21, A28, F9, G9,H2, J5, L1, and L5)	Disulfide bond formation
Phosphorylation	Kinase (F10 and B1) Substrate of F10 (G7, A30, F17, A17, and A14) Substrate of B1 (H5)	Protein phosphorylation
Dephosphorylation	Phosphatase (H1) Substrate (F17 and A17)	Protein dephosphorylation
Proteolytic processing	Proteinase (I7) Substrate (L4, A3, A10, A12, A17, and G7)	Cleave structural core proteins and membrane precursors

pathway by transferring oxidizing potential from E10 through A2.5 to G4 (147). The E10 protein is a member of the ERV1/ALR family, which utilizes the oxidizing potential of flavin adenine dinucleotide (148). The intermediate component, A2.5, forms disulfide-linked complexes with E10 and G4 (147). Oxidized G4 can then catalyze disulfide bond formation on a number of different poxvirus proteins such as A16, A21, A28, F9, H2, L1, and L5 (13, 122, 145, 147, 164, 165). Proteins of the ERV/ALR family are encoded by all poxviruses and are characterized by a ~100-residue domain that is adapted for the catalysis of disulfide bond formation in various organelles and biological settings. Proteomic studies of these VACV proteins should continue to provide insight into this biochemical pathway, which is in some respects a unique variation of the cellular electron-transporting redox pathways.

The phosphorylation and dephosphorylation of proteins also play important roles in diverse cellular and viral processes. VACV encodes two protein kinases (F10 and B1) and one phosphatase (VH1), all of which regulate the viral life cycle (Table 3) (96). During VACV morphogenesis, a number of viral proteins, namely, G7, A30, F17, A17, and A14, undergo F10-mediated phosphorylation (105, 161). Although it is not always possible to demonstrate PPIs between kinases and their substrates, physical interactions between A30, G7, and F10 have been demonstrated by immunoprecipitation (105). Studies of the early steps in virion morphogenesis have shown by immunoprecipitation that the membrane proteins A17 and A14 are phosphorylated by the F10 protein kinase (11, 44), and these PPIs are required in order to form membranes associated with immature virions (11). The VACV B1 protein contains consensus sequences present in cellular serine/threonine kinases, and H5 was shown to be phosphorylated at Thr-84 and Thr-85 by B1 (8, 19). On the other hand, the VH1 phosphatase was shown to be capable of dephosphorylating F17 and A17, which allows reversible phosphorylation (44, 96). The VH1 phosphatase was also shown to be encapsidated within the virion, and the absence of this viral protein results in reduced infectivity, even though the generation of viral particles was not affected.

Another key step in the life cycle of most viruses involves the proteolytic processing of viral proteins by a virus-encoded protease. In 1970, VACV was shown to proteolytically process its structural proteins (83). During morphogenesis from IV into MV particles, the VACV core structural proteins and membrane precursors are proteolytically processed by the viral I7 proteinase (2, 21). I7 was believed to be a cysteine protease due to its high sequence similarity to proteinases from both African swine fever virus and adenovirus. Mutational analysis revealed that I7 cleaves the substrate viral proteins at a conserved AG/X sequence, where X has a preference for a small residue such as alanine, serine, or threonine (2). Immunological methods were utilized to identify the known substrates for I7, which include L4, A3, A10, A12, A17, and G7 (2, 20, 105, 175). Since I7 is involved in the cleavage of distinct viral proteins at different stages of virus assembly, I7 represents an attractive target for antiviral drug development.

The analysis of which virion components are involved in virus attachment and entry also provides another avenue for the development of targeted antiviral therapies for human pathogens. A specific cellular entry receptor for VACV has not been identified, and the promiscuous ability of poxviruses to bind and enter most mammalian cells suggests that the cellular determinants for poxvirus entry must be very ubiquitous (102). However, VACV encodes multiple proteins involved in mammalian cell membrane fusion and entry (111). Virus attachment, the activation of fusion proteins, and membrane fusion represent the general steps for enveloped virus fusion (51). Enveloped viruses generally enter cells though the plasma membrane at a neutral pH or through endosomal vesicles at a low pH. VACV WR has the capacity to enter most mammalian cells via either route, but there are significant variations between VACV strains and cell types (9). Proteomic studies have identified the components of the VACV EFC by immunoprecipitating epitope-tagged viral proteins to capture the complex by immunoaffinity. The proteins present in the EFC were identified by MS, and their identification was confirmed by Western blot analysis. The poxvirus EFC consists of at least eight virus-encoded subunits that fall into two groups, as shown in Table 4 (144). One group consists of A21, A28, G3, H2, and L5, each of which contains an N-terminal transmembrane domain and zero to two intramolecular disulfide bonds. The other set of proteins consists of A16, G9, and J5, which are orthologs containing a C-terminal transmembrane domain and 3 to 10 predicted disulfide bonds (144). These proteins are conserved in all poxviruses, which suggests that their functions are nonredundant and essential. Recently, coimmunoprecipitation studies confirmed that F9 and L1, which are also required for

TABLE 4. Protein complexes of VACV related to entry and fusion

Protein complex	VACV proteins	Function
Poxvirus EFC	Components consisting of A21, A28, G3, H2, L5, A16, G9, and J5 EFC-associated proteins L1 and F9	Virus-cell fusion; low-pH-induced cell-cell fusion and neutral-pH cell-cell fusion depend on EFC
Fusion regulatory proteins	A56 and K2	Prevent fusion by direct interaction with the EFC
A17-A27 fusion complex	A17 and A27	Cell fusion
A26-A27 complexes	A26 and A27	Partially suppress cell fusion

cell entry and membrane fusion, interact with the EFC (13, 18).

The poxvirus regulatory fusion proteins A56 and K2 have been shown to form a complex in the EV and at the cell surface by immunoprecipitation (166) (Table 4). A56 encodes hemagglutinin that can be anchored in the EV and the infected-cell plasma membrane and is known to interact with elements of the complement pathway (62). A serine protease inhibitor (SPI-3) encoded by K2 depends on the association with A56 for membrane localization (166). By direct interaction with the poxvirus EFC, the A56/K2 complex prevents virus entry and fusion and likely mediates the phenomenon of superinfection exclusion (167–170). Additional viral PPI studies have shown that both A16 and G9, which are physically associated within the EFC, are required for binding to A56/K2 (170).

Recent studies of the A17-A27 and A26-A27 fusion complexes determined that A17, A25, A26, and A27 can be copurified in virions (71). A26 is an enveloped protein of the intracellular MV. Immunoprecipitation experiments to investigate these complexes have shown that A26 forms a disulfide-linked complex with A27 prior to virion assembly. This interaction is independent of other viral proteins and directs A26 to the MV membrane surface through A27 (28, 70). Another protein, A17, has been shown to bind to the C-terminal α-helix of A27 and, as a protein complex, induces pH-dependent cell fusion (88, 166). The association of A26 with A17 is mediated by A27. The interaction of A26 with A27 occurs through the C terminus of A26, which is implicated in playing a role in cell attachment (29). The physical association of the two membrane attachment proteins A26 and A27 partially suppresses glycosaminoglycan-mediated cell fusion (28). A25 is a truncated form of the cowpox virus (CPXV) A-type inclusion (ATI) matrix protein. Previous work has shown that A25 is required for the formation of the ATI and that A26 is needed for the attachment of virions to the ATI (103). The association of A26 with A25 supports a direct role of A26 in the embedding of virions within the ATI. These proteomic studies have thus provided a better understanding of the components involved in poxvirus attachment and fusion.

VACV encodes a number of interactive viral proteins that play a role in transcription and genome replication. The networks of binary viral PPIs involved in VACV replication and transcription are summarized in Fig. 1A. VACV replication/transcription occurs entirely in the cytoplasm of infected cells; therefore, the viral genome encodes the majority of viral proteins needed for this process, although some host ancillary proteins are recruited for intermediate and late gene transcription (110). A genome-wide Y2H screen showed that VACV A20 (DNA polymerase processivity cofactor) interacts with D4 (uracil DNA glycosylase), D5 (nucleic acid-independent nucleoside triphosphatase) (45), and H5 (DNA binding protein) (101). Furthermore, VACV A20 and D4 form a heterodimeric processivity factor by associating with E9 (catalytic subunit of the DNA polymerase holoenzyme) to comprise the processive DNA polymerase holoenzyme (157). The stoichiometry of binding for the three VACV proteins was determined to be 1:1:1 (87, 157). The regions of A20 required for binding to D4, D5, and H5 were determined and do not overlap. The binding domain of A20 for D5 maps between amino acids 26 and 76, and the minimal region of A20 for H5 binding is between amino acids 201 and 251 (76). The A20 binding site on D4 mapped to the N-terminal 25 residues of D4 (76). N-terminal residues 1 to 16 and C-terminal uracil DNA glycosylase residues 208 to 218 of D4 may also contribute to the interaction with A20 (141). Structural analysis supports a model with A20 functioning as a central scaffold protein with separate binding regions for D4, E9, D5, and H5 (141). VACV proteins G2, A18, and H5 also interact with each other in vitro and in vivo (16). Studies of these interactions have shown that G2 is a positive transcription elongation factor (15). H5 has been shown to play a role in several key steps in viral replication (39) including DNA synthesis, late gene transcription, and virion morphogenesis. It was also determined that the A18 DNA helicase functions as a transcript release factor and a postreplicative negative transcription elongation factor (94, 178). The association of G2, A18, and H5 may form a complex with other viral and/or cellular factors that possess both negative and positive elongation activities to control intermediate and late gene transcription and elongation in VACV. The interactions between E9 and A20, H5, G2, and A18 may also play a role in regulating the transition from early to intermediate and late gene expression. Four VACV late transcription factors, A1, A2, G8, and H5, interact with each other, as illustrated in Fig. 1A (42). The association of the viral RNA polymerase-associated factor H4 and D11 nucleoside triphosphate phosphohydrolase I (NPHI) was shown to be required for early gene transcription termination, explaining the known restriction of signal- and factor-dependent termination to early genes (107, 129). In addition, study of the association between poly(A) polymerase J3, viral RNA polymerase-associated factor H4, and D11 suggests that this interaction may serve as a docking site for J3 in RNA processing (106, 107).

Virion morphogenesis is a complicated process, and studies have focused on identifying the specific proteins that are involved in this complex cytoplasmic process and their functional roles. To identify early events in morphogenesis, VACV expressing V5 epitope-tagged F10 protein kinase was generated. F10 kinase was selected because previous studies demon-

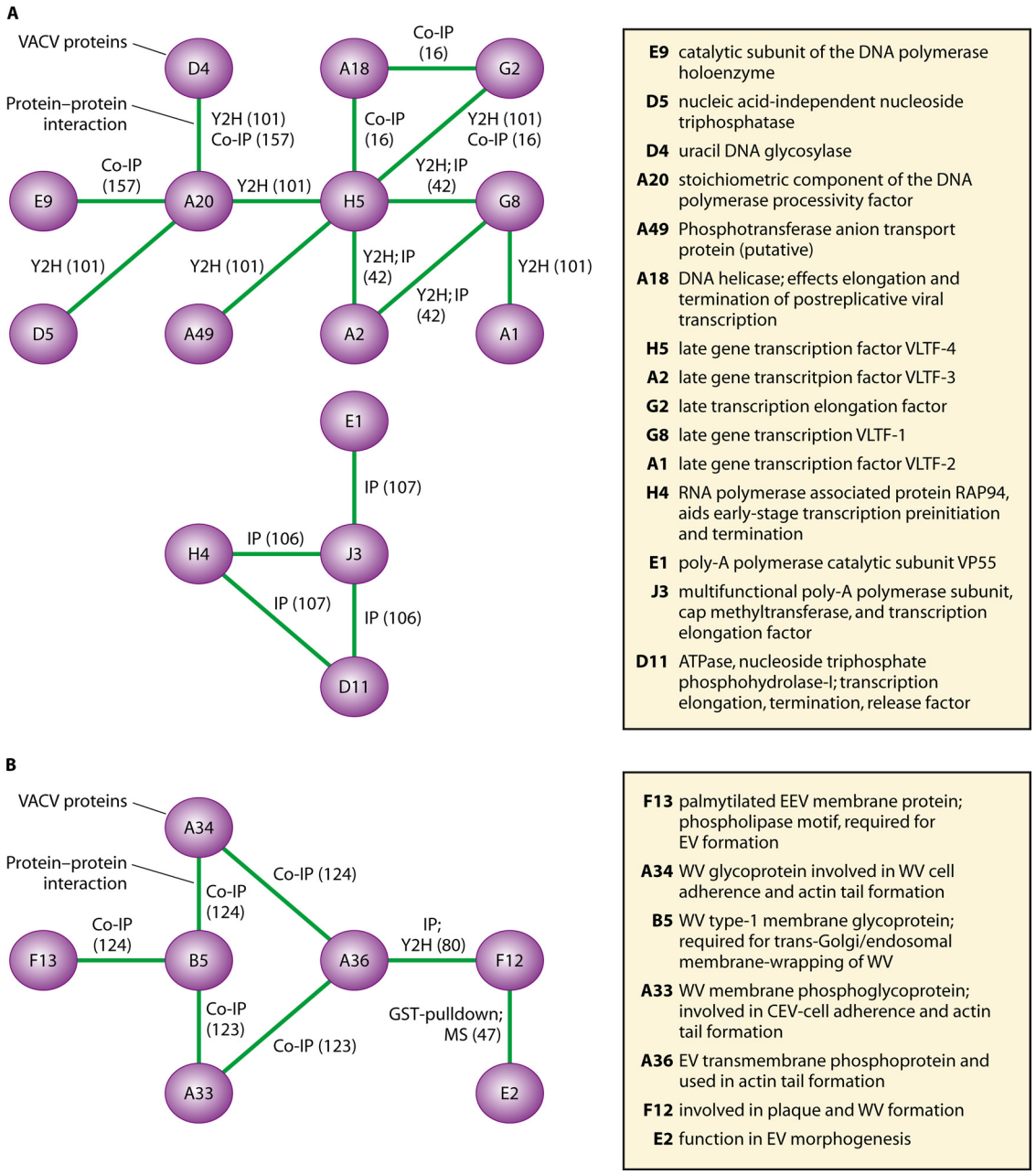

FIG. 1. Diagrammatic representation of VACV PPI networks. (A) Viral PPIs involved in VACV DNA replication and transcription. (B) Viral PPIs involved in VACV WV morphogenesis. In the interactome network, a circle represents a VACV protein, and a line represents a PPI. Inside the circle is the name of the VACV protein. Methodologies used to determine the specific PPIs are coimmunoprecipitation (Co-IP), immunoprecipitation (IP), Y2H, and MS. VLTF-4, viral late transcription factor 4; EEV, extracellular enveloped virus; CEV, cell-associated extracellular enveloped virus.

strated that it is required for the assembly of viral membranes (11). Immunoaffinity purification of the V5-tagged F10 protein from an infected-cell lysate, followed by MS, enabled the identification of a seven-protein complex (F10, A30, G7, A15, D2, D3, and J1), which is rigorously conserved in all chordopoxviruses. VACV undergoes complex morphogenesis, which involves three virion forms: IV, MV, and WV. This seven-protein complex is required for the association of membranes and viroplasm in order to form the IV that then initiates the assembly of MVs and WVs (161).

VACV proteins that are associated with the two infectious forms of the virus, MV and WV, have also been studied. Studies of viral protein interactions identified nine surface proteins in the MV, including A33, A34, A36, B5, F12, F13, E2, K2, and A56 (31). It was previously shown that the deletion of any of these viral genes (except those encoding K2 and A56)

gives rise to a small-plaque phenotype, indicating a reduced ability of cell-to-cell spread (31). Furthermore, B5 and F13 are required for the efficient and complete wrapping of MV, whereas A36 and F12 are required for the microtubule-mediated transport of WV to the cell surface. Four proteins, A33, A34, A36, and B5, are involved in actin tail formation, and all of these proteins except A36 are involved in the release of extracellular WV (17, 67, 84, 85). Among the WV proteins, multiple PPIs have been identified (Fig. 1B), which should either determine their localization in the wrapping membranes or contribute to WV transport, egress, and the induction of actin tails. Residues 91 to 111 of A36 and the cytoplasmic tail of A33 are involved in a particularly high-affinity PPI (173). The incorporation of the VACV A36 protein into the outer membrane of WV depends on the expression of the A33 protein. Thus, the A33 protein directs the A36 protein to the WV membrane, where it subsequently becomes tyrosine phosphorylated as a signal for the formation of an actin tail that facilitates egress and spread (177). To induce actin tails at the cell surface, an outside-in, B5-dependent signaling event triggered by membrane-bound EV is thought to be required (117). The coexpression of A33 with B5 resulted in the colocalization of the two proteins in noninfected cells. The transmembrane domain of B5 is the major determinant of the interaction with A33 (123). Since no direct interaction between B5 and A36 has been found, a possible role for the A33-B5 interaction is to incorporate A36 into the complex. The lumenal domains of A34 and B5 are sufficient to mediate their interaction (50, 124). This interaction is required for the proper targeting and subsequent incorporation of B5 into EVs since the amount of B5 is greatly reduced in EVs formed in the absence of A34 (50). The PPI between A34 and A36 has been studied (124, 138). The targeting of A36 to WV is dependent on the presence of A34 (124). An interaction between F12 and A36 was shown to be critical for the function of F12 during viral egress (80). The association of F12 with E2 is necessary for MV morphogenesis prior to its microtubule-based transport toward the plasma membrane (47). The interaction between B5 and F13 is suggested by their colocalization (75); however, no direct biochemical PPI evidence has been reported. These studies highlight viral protein interactions that play important roles in the virion morphogenesis of VACV and likely all other poxviruses as well.

GLOBAL PROTEIN EXPRESSION STUDIES

The vaccination of individuals with VACV was a major factor contributing to the success of the smallpox eradication campaign; however, the immunological basis for protection against smallpox was largely unknown and even today is not well understood. To begin to address this issue, a VACV protein chip assay was developed to characterize the humoral response of humans who were vaccinated with VACV or had recovered from smallpox infection (37). An advantage of these global protein expression studies is that they are capable of providing data in a more-high-throughput parallel fashion than individual immunoprecipitation studies. For the VACV protein chip assay, high-throughput cloning of individual VACV WR genes was accomplished by PCR amplification of the individual ORFs, which were then cloned into a T7-based plasmid expression vector by homologous recombination. Individual VACV proteins were expressed using an *Escherichia coli*-based cell-free transcription/translation system and then printed onto the protein chip. The protein chip was then probed with either archival sera from an individual convalescing from smallpox or sera of individuals vaccinated with the Dryvax VACV vaccine, and the amount of captured antibody was quantified by using fluorescent secondary antibodies.

The probing of whole-proteome microarray chips generates an antibody profile against a subset of all the antigens in the proteome that may be useful for diagnostic purposes as well as for the production of subunit vaccines. In this regard, one study reported a trend toward the recognition of antigens with late temporal expression, while early genes were underrepresented (37). Additionally, viral structural proteins and proteins with transmembrane domains were predominant compared to the viral proteome as a whole. The immunoglobulin G (IgG) profiles showed considerable interindividual heterogeneity in human IgG profiles, and this has also been seen for other large, complex pathogens (37). Viral antigens recognized by all individuals tested were surprisingly uncommon. IgG responses to H3 (an envelope protein found on mature virions), A10 (major core protein), and A25 (the ortholog of the CPXV ATI protein) were seen in all 13 individuals tested for a primary response to Dryvax, while only H3 and A10 were recognized by 12 individuals after boosting. A total of 14 antigens were recognized by over half of all 25 individuals tested, whereas 52 antigens were recognized by less than half of the individuals tested. Despite heterogeneity, a subset of commonly recognized antigens (the membrane proteins A13, A17, A27, A33, A36, B5, D8, F13, H3, and L1; the core proteins A10, I1, and L4; and other proteins, A11, A52, B2, E2, D13, G5, H5, and A25) was identified, and these antigens provide the best candidates for the development of subunit vaccines and for the development of diagnostics. The VACV antigens printed onto the protein array also allowed the detection of antibodies against conserved epitopes in variola virus (VARV), providing evidence that protection by VACV against VARV is due to serological cross-reactivity.

In another study, the immunological responses following vaccination with VACV-WR, Dryvax, and modified VACV Ankara (MVA) were compared by using VACV-WR protein microarrays and sera from rabbits, macaques, and humans (38). MVA is an attenuated, nonreplicating variant of VACV, but it has been shown that the majority of viral structural genes are intact and that the ability to protect animals against orthopoxvirus challenge has been retained (3, 127). Therefore, the goal of that study was to evaluate the effect of the many mutations and deletions in MVA on the antibody response and to determine the correlation between the macaque and human profiles in response to both vaccines in order to evaluate the macaque as a model for virus infection in humans. The immune response profiles for MVA and Dryvax were similar for both humans and macaques. Antibodies to MV and EV forms of virus were detected, while the responses to nonmembrane proteins were less well conserved. A primary difference in MVA and Dryvax was the lack of a response to the immunodominant ATI protein homolog WR148 in the profile for MVA, which is expected because the gene is deleted in MVA. In rabbits, an expansion in the response to nonstructural and

early proteins was detected in the WR profiles compared to MVA; however, the membrane protein responses were similar. There was a high level of concordance between the human and macaque immune profiles, which suggests that the macaque is a good model for the immune response in humans and that MVA should provide protection against lethal orthopoxvirus challenge (38). This work demonstrates the power and utility of protein chip technology, but note that the correct folding of the query viral proteins at each spot remains a problematic issue.

In the past, functional studies of poxvirus proteins have been hampered by a lack of sufficient reagents, such as purified proteins and antibodies. To address the need for generating larger quantities of viral proteins for subsequent studies, a high-throughput system was developed for generating recombinant bacmids and baculoviruses that overexpress individual VACV proteins. Viral genes were PCR amplified from complete viral genomic DNA, and specific restriction sites were included for the purpose of cloning between these unique restriction sites in modified plasmids such that the expressed proteins were fused to an N-terminal hexahistidine tag for use in purification. Recombinant baculoviruses were produced and tested for protein expression in insect cells, and proteins were purified on Ni-nitrilotriacetic acid resin. A high-throughput method for baculovirus expression in 24-well blocks was developed, as was a high-throughput procedure for protein purification, and proteins were then assayed for solubility. Of 78 VACV proteins tested, 62 genes (79%) were expressed in this system, and this will allow the production of antibodies against these proteins and more detailed physical studies (60).

STRUCTURAL PROTEOMICS

Increased concern over the potential use of VARV as a biological weapon and the recent outbreak of MPXV in the United States have emphasized the need for structural studies of poxvirus proteins in order to develop new antiviral drugs and to better understand poxvirus interactions with the host. Also, CPXV infections are becoming more common in Europe, and there are several cases of live VACV vaccine reentering new animal reservoirs in several parts of the world such as India and Brazil (113). One practical goal of these studies is to obtain valuable structural information for the identification of small-molecule inhibitors or drugs that can be developed into the next generation of poxvirus-specific antivirals (115). Currently, the Protein Data Bank (PDB) (http://www.pdb.org/) contains approximately 75 structures of poxvirus-encoded proteins, more than half of which were solved in the last 5 years (Table 5). For example, the dual-specificity phosphatase encoded by VARV (H1 phosphatase) is essential for the production of mature virus particles, making this protein an excellent target for the development of antiviral drugs. Based on the crystal structure, in silico screening was performed, and several novel compounds with potential antiviral properties were identified (126). Moreover, the phosphatase encoded by VACV (VH1) has been shown to dephosphorylate STAT1, resulting in the downregulation of the cellular antiviral response (99). This viral phosphatase is inactive against STAT1 bound to DNA, suggesting that the viral protein acts predominantly on activated STAT1 in the cytoplasm, which is relevant in light of the fact that the site of viral replication occurs in the cytoplasm for poxviruses. The crystal structure of VH1 revealed a dimeric quaternary structure, which exposes two active sites spaced approximately 39 Å away from each other, and this was proposed to be essential for the specific recognition of activated STAT1 by preventing its nuclear translocation and thus blocking gamma interferon (IFN-γ) signal transduction and the antiviral response (89). Recently, the crystal structure of the ectromelia virus (ECTV)-encoded IFN-γ binding protein complexed with IFN-γ was solved, providing vital insight into the mechanism by which ECTV blocks the IFN-γ signal transduction pathway (119). An alternate mechanism to block the IFN-γ signal transduction pathway is through the binding and sequestration of interleukin-18 (IL-18). In humans, IL-18 plays a role in inflammation as well as in host defenses against microbes. The biological activities of IL-18 are normally regulated by a host regulatory protein called IL-18 binding protein (IL-18BP). Functional homologs of IL-18BP are encoded by all orthopoxviruses and contribute to virulence by suppressing IL-18-mediated immune responses. Recently, the crystal structure of ECTV IL-18BP complexed with human IL-18 was solved. Upon binding, ECTV IL-18BP blocks a putative binding site on IL-18, and this prevents IFN-γ induction by IL-18 (91). These studies are important for the rational design of inhibitors against the viral IL-18BP, which is a potential therapeutic target for the treatment of orthopoxvirus infections in general.

Many viruses, including poxviruses, express antiapoptotic proteins to counter host defense mechanisms that would otherwise trigger the rapid clearance of infected cells. Nuclear magnetic resonance (NMR) structural data for the VACV protein K7 revealed that it adopts an α-helical fold belonging to the Bcl-2 family of apoptosis regulators despite having an unrelated amino acid sequence (82). K7 forms a complex with the dead-box RNA helicase DDX3 and suppresses DDX3-mediated IFN-β promoter induction (82). The X-ray crystal structure for the MYXV M11L protein also revealed a Bcl-2 fold, although it too possesses very little amino acid sequence similarity to Bcl-2. The Bcl-2 fold of M11L allows it to associate with BH3 domains, especially those of Bax and Bak, essentially sequestering these proapoptotic factors and blocking their action (48, 92). Although other VACV proteins, such as A52 and B14, have been shown to also adopt a Bcl-2 fold (64), they are unable to modulate apoptosis since they lack the groove to bind BH3 peptides. Instead, A52 and B14 inhibit the activation of NF-κB by blocking the production of proinflammatory cytokines (64). Poxviruses express several proteins that allow for viral persistence by preventing apoptosis. For example, the E3L protein encoded by VACV has been shown to bind double-stranded RNA (dsRNA) and prevent the activation of dsRNA protein kinase (PKR), also inhibiting apoptosis (81). Another VACV protein, K3L, is a eukaryotic initiation factor 2 alpha mimic and blocks eukaryotic initiation factor 2 alpha phosphorylation and PKR autophosphorylation (36). VACV also expresses the F1L protein, which interferes with the release of cytochrome *c*, inhibiting cytochrome *c*-mediated apoptosis (93).

Orthopoxviruses also encode serine proteinase inhibitors (serpins), which neutralize active proteases by binding to them and forming an inhibitory complex. The first poxvirus serpin to

TABLE 5. Poxvirus protein structures[a]

Protein	PDB accession no.	Type of structure data	Poxvirus	Release date(s) (day, mo, yr)	Reference
vCCI (p35)	1cq3	X-ray diffraction	CPXV	12 November 1999	23
CrmA	1c8o, 1m93	X-ray diffraction	CPXV	6 September 2000, 5 August 2003	154
IFN-γ binding protein/IFN-γ complex	3bes	X-ray diffraction	ECTV	12 February 2008	119
IL-18BP bound to human IL-18	3f62	X-ray diffraction	ECTV	6 January 2009	91
EVM1 chemokine binding protein	2grk	X-ray diffraction	ECTV	9 May 2006	5
EVM053 glutaredoxin1	2hze, 2hzf	X-ray diffraction	ECTV	21 November 2006	7
M156R	1jjg	NMR, 20 structures	MYXV	06 March 2002	133
M11L (Bcl-2 mimic)	2jbx, 2jby	X-ray diffraction	MYXV	27 March 2007	92
M11L	2o42	X-ray diffraction	MYXV	6 March 2007	48
vCCI and human macrophage inflammatory protein 1beta	2ffk, 2fin	NMR, min avg; NMR, 15 structures	Rabbitpox virus	22 August 2006	181
A41	2vga	X-ray diffraction	VACV	26 February 2008	Unpublished data
A52	2vvx, 2vvw	X-ray diffraction	VACV	26 August 2008	64
B14	2vvy	X-ray diffraction	VACV	26 August 2008	64
CrmE	2uwi	X-ray diffraction	VACV	10 July 2007	63
E3L	1oyi	NMR, 20 structures	VACV	09 March 2004	81
F1L	2vty	X-ray diffraction	VACV	24 June 2008	93
G4	2g2q	X-ray diffraction	VACV	01 August 2006	160
Hemagglutinin precursor HA0	1ha0	X-ray diffraction	VACV	12 October 1998	26
K3L	1luz	X-ray diffraction	VACV	28 August 2002	36
K7	2k36	NMR structure	VACV	28 October 2008	82
L1	1ypy	X-ray diffraction	VACV	01 March 2005	158
Fab 7D11 neutralizing antibody against L1	2i9l	X-ray diffraction	VACV	18 September 2007	159
mRNA capping enzyme D1 subunit	2vdw	X-ray diffraction	VACV	13 May 2008	41
N1	2uxe	X-ray diffraction	VACV	22 May 2007	32
N1L	2i39	X-ray diffraction	VACV	21 November 2006	4
Polyadenylate polymerase	2ga9, 2gaf	X-ray diffraction	VACV	16 May 2006	112
N-terminal 9 kDa of DNA topoisomerase	1vcc	X-ray diffraction	VACV	08 March 1996	150
Type I topoisomerase	1a41	X-ray diffraction	VACV	01 June 1999	27
Thymidine kinase complexed with TTP	2j87	X-ray diffraction	VACV	13 November 2006	53
Thymidine kinase bound to TDP; brivudin monophosphate	2v54, 2w0s	X-ray diffraction	VACV	21 October 2008	22
Tyr/Ser phosphatase	2q05	X-ray diffraction	VACV	19 June 2007	Unpublished data
Uracil-DNA glycosylase	2owq, 2owr	X-ray diffraction	VACV	24 July 2007	141
dUTPase	2okb, 2okd, 2oke, 2ol0, 2ol1	X-ray diffraction	VACV	01 May 2007	139
Complement protein	1rid	X-ray diffraction	VACV	22 June 2004	59
Complement control protein	1vvc, 1vvd, 1vve	NMR; NMR, 21 structures	VACV	03 December 1997	176
Complement control protein	1e5g	NMR, 50 structures	VACV	31 August 2000	66
VCP	1g40, 1g44	X-ray diffraction	VACV	07 February 2001	114
VCP (suramin)	1y8e	X-ray diffraction	VACV	23 August 2005	58
VH1, VH1 phosphatase, bound to xenon	2rf6, 3cm3, 3ceo	X-ray diffraction	VACV	30 September 2008, 10 February 2009	89
VP39	1av6	X-ray diffraction	VACV	25 February 1998	68
VP39	1b42, 1bky, 1eqa, 3mag, 3mct, 4dcg	X-ray diffraction	VACV	22 July 1999	73
AS11 variant VP39	1vpt	X-ray diffraction	VACV	17 August 1996	69
DC26 mutant of VP39	1p39, 1v39, 1vp9, 2vp3	X-ray diffraction	VACV	17 September 1997	70
VP39 mutant	1eam	X-ray diffraction	VACV	14 June 1999	73
VP39 F180W mutant, complex	1jte, 2jtf, 1jsz	X-ray diffraction	VACV	10 July 2002	74
VP39 complex	1vp3	X-ray diffraction	VACV	17 September 1997	70
VARV topoisomerase covalently bound to DNA, noncovalent	2h7f, 2h7g	X-ray diffraction	VARV	15 August 2006	125
H1 phosphatase	2p4d	X-ray diffraction	VARV	29 May 2007	126
Viral Z alpha domain	1sfu	X-ray diffraction	Yatapoxvirus	17 August 2004	65

[a] NMR or X-ray diffraction structures are available in the PDB at http://www.pdb.org/.

be discovered was cytokine response modifier A (CrmA) from CPXV (10). CrmA (also called SPI-2) was originally shown to inhibit IL-1β-converting enzyme (19), now known as caspase 1 (90). The role of CrmA as a viral inhibitor of inflammation was intriguing, and further studies of CrmA showed that it plays a role in inhibiting apoptosis in cultured cells (46, 86). Expressing viral proteins that inhibit apoptosis is one mechanism that poxviruses use to evade the host innate immune response at the cellular level. Studies of the X-ray crystal structure of CrmA showed that it has a structure similar to that of other serpins, even though there is very little primary sequence similarity to any one cellular serpin (153, 154). Orthopoxviruses encode three serpins, SPI-1, SPI-2/CrmA, and SPI-3. MYXV also encodes three serpins, designated SERP-1, SERP-2, and SERP-3. Although studies have shown the MYXV serpins exhibit functional similarly to those of orthopoxviruses, SERP-2 and CrmA are not interchangeable during viral infection and pathogenesis in vivo (116). These studies demonstrate

the complexity and specificity of the interactions between viral pathogens and their host.

Tumor necrosis factor (TNF) plays a role in the inflammation process and is part of the immune response to various pathogens. Many poxviruses encode secreted TNF receptor mimics that bind and sequester TNF, which prevents the activation of cellular TNF receptors and TNF-mediated responses that would otherwise result in downstream antiviral effects (130). Orthopoxviruses all express at least one viral TNF receptor mimic, called a cytokine response modifier (Crm) protein, which provides protection against cellular apoptosis by TNF. The crystal structure of VACV CrmE was solved recently and confirms that it possesses a TNF receptor fold, and binding studies have shown that CrmE has a high affinity for TNF (63).

VIRUS-HOST-INTERACTING PARTNERS

PPIs play a pivotal role in all biological processes. The identification of novel interactions between viral and host proteins can provide valuable knowledge regarding the possible mechanism(s) by which these viral proteins contribute to poxvirus replication and host subversion. Therefore, the mapping of virus-host PPIs provides a framework for an understanding of the functional role(s) of proteins implicated in various cellular processes such as apoptosis, cell cycle control, the formation of the cytoskeleton, and innate immune responses, among others. Despite the availability of newer PPI methodologies that will be described below, the Y2H screening system remains one of the most practical and efficient techniques used to identify large numbers of pairwise interacting protein partners (40, 77).

Recently, Myriad Genetics (http://www.myriad.com) has developed an automated robotic process for the high-throughput identification of poxvirus-host PPIs by using Y2H as a platform (40). The Y2H technology, which was pioneered by Fields and Song in the late 1980s, uses test proteins (or baits) to screen for interactions against a large group of randomly cloned protein libraries (or preys). A protein referred to as "bait" is fused to a promoter-specific DNA binding domain, and a second protein referred as "prey" is fused to a transcriptional activator domain. During the interaction of the bait and prey proteins, which occurs exclusively in the yeast nucleus, the DNA binding and activator domains are placed in close proximity so that the expression of a reporter gene is initiated (56). Gene transcription can be monitored by using reporter genes that enable yeast to grow on selectable media or can be detected by colorimetric assays. Some of the advantages of using Y2H technology are that it allows the high-throughput screening of PPIs from higher eukaryotes in addition to being very sensitive, which facilitates the identification of proteins with low-level expression or transient interactions, like those involved in signaling events (56, 77, 171).

By using automated Y2H technology, Myriad Genetics has generated a compilation of novel poxvirus-host PPIs (183). A total of 195 human proteins from a collection of four human cDNA libraries were identified as being putative binding partners for 33 different VACV proteins (Table 6). Interestingly, none of these PPIs were previously documented in the literature. This data set provides new avenues to explore the role of previously unknown host pathways implicated in virus replication, tropism, and pathogenesis. Surprisingly, the previously well-documented PPIs between human PKR and either VACV K3 (VACV-WR034) or VACV E3 (VACV-WR059) were not identified in this screening. This was somewhat unexpected since in 1998, Sharp and coworkers used a similar Y2H technology to study the interactions between these proteins (151, 152). Therefore, results obtained with this Y2H method need to be interpreted with caution. In this regard, one well-known problem of Y2H technology is the significant number of "false positives" that are generated when proteins interact nonspecifically in the yeast nucleus (33). In fact, one drawback of the traditional Y2H assay is that the two proteins have to be directed to the nucleus. The bait and query proteins may not normally interact because they are localized in different cellular compartments in virus-infected cells; however, in the Y2H assay, the proteins are brought into close proximity and are allowed to interact, thereby sometimes giving a false positive result. Therefore, a validation of the Y2H results using additional independent PPI methodologies is required. Additionally, certain proteins have specific structural or functional requirements in order to interact with their true target partner proteins. When these requirements are not fulfilled, the interaction is prevented, resulting in a "false negative" result for interacting partners that indeed do interact in vivo. Some PPIs are also mediated by other proteins or cofactors or sometimes require specific posttranslational modifications such as phosphorylation as a prerequisite to interact. These types of interactions would not be detected by a standard Y2H assay. But, despite all of these caveats, Y2H results can indeed provide abundant clues about novel virus-host PPIs.

Although viral genomes are highly conserved among orthopoxviruses, e.g., more than 90% of major ORFs share a high level of similarity to those from VACV, a unique set of genes is specifically expressed by individual members, such as VARV. A selected Y2H search that focused exclusively on identifying VARV-specific human protein interactions that would have no direct counterparts from VACV was conducted (108). A set of 14 VARV-specific ORFs (D8L, C18L, A27L, A39L, B4L, B9R, B10R, B11R, B14L, B19R, B20R, B22R, G1R, and G2R) were included in this screen against several human cDNA libraries (tonsil, breast cancer, prostate cancer, and spleen) (Table 7). Of the 14 VARV proteins tested, only three, B22R, G1R, and G2R, revealed consistent interactions with known human proteins (108). Moreover, in that same study, Mohamed et al. confirmed the interaction between VARV G1R and both NF-κB1 and Skp1A and demonstrated the ability of VARV G1R (or closely related family members from pathogenic orthopoxviruses) to inhibit NF-κB signaling in transiently transfected human cells (108).

In addition to the VACV genome, a select few MYXV genes were also screened against different human libraries for putative binding partners by Y2H assay. In one particular case, a total of 13 potential human binding partners were identified for the MYXV host range factor M-T5 (S. J. Werden et al., unpublished data). Interestingly, neither of the two previously identified host binding partners for M-T5, namely, cullin-1 (79) or Akt (172), were identified in this Y2H screen; however, the Skp1 component of the host SCF (Skp1, culling, F-box-containing complex) ubiquitin ligase complex (that includes cullin-1) was picked up in this Y2H screen and is probably the

TABLE 6. Myriad Y2H screening results for interacting VACV and human proteins[a]

Viral protein (bait)	Host protein(s) (prey)
VACV-WR001 (C23)	EIF2S2, HNRPAB(332), KRT4, PHLDA1, PPIG, RL21, UBC, ZC3H11A
VACV-WR002	CRYAB, DNAJB6(241), HSPB1
VACV-WR019 (C9)	LSM4
VACV-WR021 (C7)	YAF2
VACV-WR022 (C6)	APIGBP1(1314), AP2B1(937), AUP1, ATP5F1(294), BRMS1(246), CDC42BPB, CEP110, CGRRF1, CHERP, CTNNA1(906), CTNNBBIP1, EXOC7(684), FYCO1, GPS2, KLC3, KRT4, MBD2(411), MYH14(1995), NEXN, OFD1, PDCD6IP, PDE4DIP(2240), RAD50(1312), RANBP2, RMP(535), RUNDC1, SEPT6(427), SEPT8(440), SFPQ, SLK(1152), STRN, SYNE2(6885), TCHP, TNNI2, TRIM11, TTN(27051),USF2(279), VASP, XPA
VACV-WR026 (C2)	CRYAB, HSPB1
VACV-WR029 (N2)	AHNAK2, AP2B1(937), DEAF1, EPS8L2, GOLGA2, KPNA2, KPNA5, MFSD3, MYOM1(1685), PLSCR4, PRDX4, R3HCC1, RAI14, SMARCC2(1214), SON(2386), TK1, TXNDC4, VCPIP1
VACV-WR034 (K3)	CIR, IL-32(234), LUC7L(325), LZTS2(669), PALMD, RPS27A
VACV-WR039 (K7)	FLJ10254, ARNTL(625), COBLL1, LAD1, NCOR1, RILPL1, RL2A, SNX5, TSPYL2
VACV-WR043 (F4)	EIF4G2, ENAH(570), GPS2, LAMC2(1111)
VACV-WR049 (F10)	FAM21C
VACV-WR059 (E3)	DHX9, MAST1, MAST2, MAST4(2137), STAU1(577), STAU2(479)
VACV-WR063 (E7)	TRAPPC5
VACV-WR064 (E8)	ATP6V0C, C6ORF166, CCDC80, CD2BP2, CRYAB, CTTNB1, GTF3C3, HLA-B(362), JUP, KHDRBS1, KIAA0256, KIF2C, LARP7(565), LPLUNC1, LRIG1, MBTD1(410), MYOZ1, NPM1(294), OBFC2A, OTG1, P2RX5(422), PDIA6, RAB11FIP5, RBM12B, RNF11, TPD32(184), UBE3A(875)
VACV-WR065 (E9)	KIAA0256
VACV-WR073 (I4)	APPBP2, C6ORF58, CAND1, CIT(2054), FOXP1(677), GOLGA2, HSPB1, LOC124220, RAI14, ZFPM2
VACV-WR084 (G6)	APPL2, COL4A3(1494), CWF19L2, DUSP1, HLA-DRA, HYPK, LAP3, LCN1, LMO7(728), MATN2(956), NUS1, QSCN6(747), RPS23, S100A7, SBSN, SON(2386), ST8SIA4(359), TCHP, TNNI1
VACV-WR085 (G7)	PRKAR2A
VACV-WR094 (J2)	PDLIM1, TK1, ZNF217
VACV-WR106 (D1)	NOMO1, NUP62
VACV-WR110 (D5)	ABHD5, ANXA1, ANXA2(339), COL6A3(3177), COPZ1
VACV-WR141 (A20)	LMNB1, LMNB2
VACV-WR142 (A22)	NUDT5
VACV-WR150 (A27)	BAP1, KRT4, TPM1(284), UNC84B(717)
VACV-WR171 (A45)	CEP135, SMAD1, TNIP2, TRIP11, UTRN
VACV-WR174 (A48)	BIN1(475), C19ORF60(180)
VACV-WR195 (B13)	MIA3(1907), SLK(1152)
VACV-WR196 (B14)	CDR2, KIAA0323, N4BP1
VACV-WR209	EGLN1(426), F8(2351)
VACV-WR227	ATP2A3(999)
VACV-WR242	CAMLG(296)
VACV-WR258	SET(290)
VACV-WR282	STAT4

[a] Adapted from www.poxvirus.org/ with permission. See also http://proteinbank.vbi.vt.edu.

direct binding partner that links M-T5 to the host SCF complex.

Yaba monkey tumor virus (YMTV) is a member of the genus *Yatapoxvirus* and can cause distinct epidermal histiocytomas (cutaneous benign skin tumors) of the head and limbs in nonhuman primates (49). YMTV 12L is an ortholog of VACV K3L, which has been studied extensively not only for its interaction with PKR (152) but also for the significance of its biological functions in evolution (52). Y2H screening was used to identify potential binding partners of YMTV 12L, and two new interactions were identified (Table 7), but PKR was not identified in this screen. The biological significance of these new PPIs has not been further examined.

The systematic Y2H study by Myriad Genetics was the first viral genome-wide screen designed to identify cellular PPIs with an entire poxviral proteome. The ability of poxvirus proteins to interact with their cellular binding partners has a major impact on the outcome of viral infection (102). Poxviruses have the specific advantage that their genomes are large and encode a vast array of factors, several of which have been demonstrated to specifically target and manipulate important biological processes in infected cells (55, 142, 182). However, the mechanisms by which most of these PPIs actually function during viral infection remain largely unknown. To address these questions, a small but increasing number of studies have employed proteomic methods to screen individual poxviral proteins for host binding partners. For example, Y2H analysis revealed an interaction between the host range protein CP77 of CPXV and HMG20A. It was further demonstrated that CP77 promotes the dissociation of the cellular protein HMG20A from the viral genome during viral replication (72). Another VACV host range gene, E3L, was reported to bind and sequester dsRNA in the cytoplasm following viral infection, subsequently blocking both the stimulation of PKR and the activation of 2′-5′-oligoadenylate synthase (24, 25, 136). Furthermore, the nuclear protein SUMO-1 (also known as PIC-1, sentrin, or GMP-1) and the cytoplasmic ribosomal protein L23a were identified as being putative binding partners of

TABLE 7. Major VARV (strain Bangladesh) and YMTV interactions with human proteins identified by myriad Y2H screening of human cDNA libraries[a]

Virus (bait)	Host protein (prey) (definition [no. of amino acids])
VARV-BSH B22R	MCC (mutated in colorectal cancer [829])
	PLK3 [Polo-like kinase 3 (646)]
	PPP1R9B (protein phosphatase 1, regulatory subunit 9B [817])
VARV-BSH G2R	CAMLG (calcium-modulating ligand [296])
	MUC5B (mucin 5B, oligomeric mucus/gel forming)
	SLC39A7 (solute carrier family 39, member 7)
VARV-BSH G1R	NFKB1 (nuclear factor of kappa light polypeptide gene enhancer in B cells 1)
	SKP1A (S-phase kinase-associated protein 1A, isoform b [164])
YMTV g12L	PKM2 (pyruvate kinase, muscle, isoform 1 [531])
	RAB11FIP3 (RAB11 family-interacting protein 3 class II, isoform [756])

[a] Numbers in brackets represent the number of amino acids present in the protein to allow the identification of the isoform identified. Data shown were reported previously by Mohamed et al. (108) and are our unpublished data (for YMTV).

E3 by Y2H assay; however, the biological significance of these interactions have not been explored (137). In a separate study, a Y2H screen was performed to identify cellular binding partners for a 68K ANK protein encoded by MVA; interestingly, 99% of the positive interactions analyzed were with cellular Skp1 (156). Not surprisingly, the 68K ANK protein of MVA contains a C-terminal F-box-like domain, which was previously documented to bind Skp1 (104). More recently, a screen of a human brain cDNA expression library by Y2H screening demonstrated that the VACV neurovirulence factor N1L interacts with human brain-originated cellular basement membrane-associated chondroitin sulfate proteoglycan (bamacan) (109). Additional experiments suggest that this virus-host PPI plays a positive role in promoting viral growth and perhaps contributes to the virulence of VACV in neural cells (109). Finally, a Y2H screen was used to investigate protein binding partners of VACV ligase (the A50R gene), and one of the nine host proteins identified was human topoisomerase II (95). This particular interaction was further confirmed by coimmunoprecipitation methods that showed that native ligase and a Flag-tagged recombinant protein form complexes with human topoisomerase II in virus-infected cells (95). Knowledge acquired from mapping of the host-virus interactome of poxviruses should continue to provide valuable insights into the mechanisms by which these proteins function within the cellular environment and may also lead to the generation of novel antiviral therapies.

NEW TECHNOLOGIES AND METHODOLOGIES

New high-throughput technologies for genome-wide expression studies have been developed and are being utilized for proteomic studies of large pathogens. One of these methodologies is nucleic acid programmable protein array (NAPPA), which is commonly referred to as an in situ protein chip assay (131, 132). Unlike the bacterially expressed proteins used for the chip assay described above to identify the components of the virion recognized by the host humoral immune response, in NAPPA, the viral proteins are expressed de novo on the chip. The DNA expression vectors on each spot are biotinylated and spotted onto the slide with avidin plus a capture antibody that recognizes the common epitope tag on all of the viral target proteins. The tagged viral proteins (for example, glutathione *S*-transferase [GST] fusions) are expressed following the transcription/translation of the expression plasmids using a cell-free system (e.g., reticulocyte lysate) and then immobilized by a capture monoclonal antibody to the epitope tag, as shown for

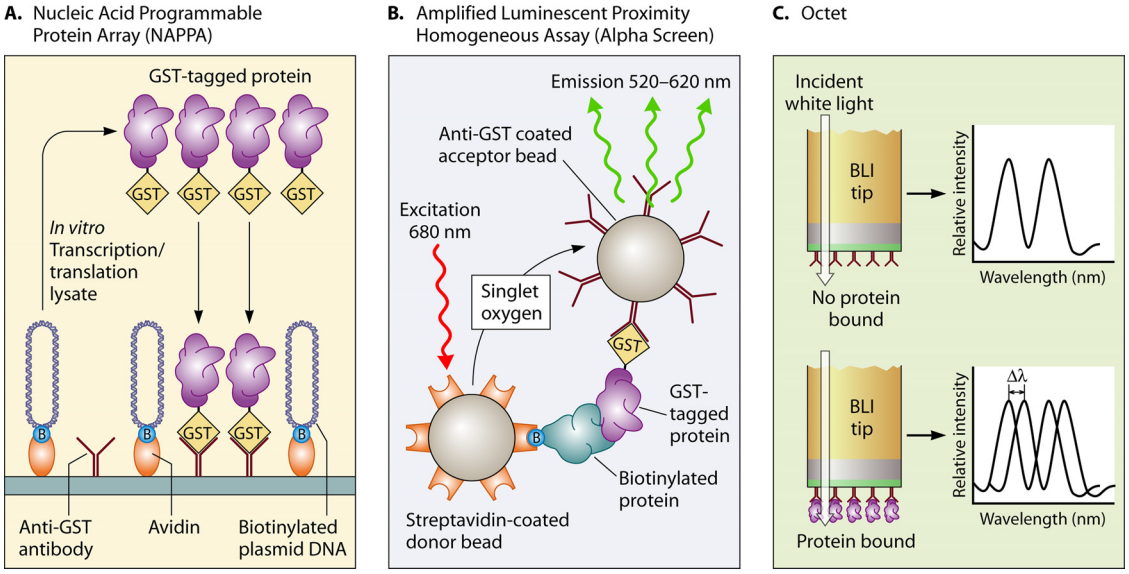

FIG. 2. New technologies for high-throughput proteomics. (A) NAPPA. (B) Amplified luminescent proximity homogeneous assay (Alpha screen). (C) Octet technology. BLI, biolayer interferometry.

GST fusions in Fig. 2A. Specific PPIs can be detected in this system by the combined expression, within the same extract, of a query host protein fused to a second tag, and an antibody to this second tag is used to probe the array. Thus, the query protein has an alternative epitope tag, and the interaction of the query host protein with any of the target viral proteins on the array can be detected by using an antibody to the epitope tag fused to the query protein (131, 132). NAPPA allows the high-throughput analysis of one query protein against hundreds or thousands of target proteins simultaneously, which is particularly useful for studies of large and complex pathogens.

High-throughput methodologies for studies of PPIs require validation to verify that the interactions are specific and occur within virus-infected cells. Current technologies that can be utilized for the validation of specific PPIs include traditional methods like coimmunoprecipitations, GST pulldowns, and technologies linked to confocal microscopy, such as fluorescence resonance energy transfer and biolumenescence resonance energy transfer. More recently, several newer technologies have also been exploited, such as the amplified luminescent proximity homogeneous assay (Alpha screen; Perkin-Elmer Life and Analytical Sciences, Waltham, MA) and Octet technology (Forte Bio, Menlo Park, CA). The Alpha screen technology is similar to that of the protein chip assay, but it can also be used to confirm putative PPIs that have been identified by using other methods. In the example shown in Fig. 2B, one expressed protein is biotinylated, while the other expressed protein possesses a GST tag. Streptavidin donor beads and anti-GST acceptor beads are added and allowed to bind to the expressed proteins. If the two proteins interact, they will be brought into close physical proximity such that when the donor bead is excited at 680 nm by an excitation laser, singlet oxygen will be released, and if they are within less than a 200-nm proximity, this will be detected by the donor bead emission at 520 to 620 nm. If the proteins do not interact, the acceptor and donor beads will not be in close-enough proximity to generate emission light. Alpha screen technology can be used to validate PPIs identified by using various proteomic techniques such as NAPPA, Y2H, and coimmunoprecipitation studies.

Another newly developed method for investigating PPIs is the technology used in the Octet system (ForteBio, Inc., Menlo Park, CA). The Octet system uses biolayer interferometry, which is an optical analytical technique that analyzes the interference pattern of white light reflected from two surfaces, a layer of immobilized protein on the biosensor tip and an internal layer (128). If there is a change in the number of molecules bound to the biosensor tip, this causes a shift in the interference pattern that can be measured in real time. When an immobilized ligand bound to the biosensor tip binds with a binding partner, this results in an increase in optical thickness at the sensor tip, which results in a shift in the wavelength of the reflected light (Fig. 2C). Any nonbound proteins that do not interact with the immobilized protein of interest on the tip do not affect the reflected light interference pattern. Therefore, Octet technology is unique in that biolayer interferometry can be used with crude unpurified samples to evaluate PPI, affinity, and kinetics. Rapidly developing biosensor technologies (1, 135) will advance proteomic studies of PPIs in general.

Surface plasmon resonance (SPR) technology such as Biacore (GE Healthcare, Piscataway, NJ) is an affinity-based biosensor system that has been widely utilized to investigate PPIs in vitro (128). In SPR, one of the reactant molecules (ligand) is covalently or noncovalently bound to the chip surface, which uses microfluidics to flow the other reactant (analyte) over the chip. This type of biosensor detects binding events using SPR to measure the refractive-index changes that occur due to the analyte binding to the molecule on the surface of the chip. Initially, it was demonstrated that proteins could be immobilized reversibly to a Ni-nitrilotriacetic acid Biacore chip using a His-tagged VACV protein, His_6-VP55. The VACV proteins VP55 and VP39 were known to interact, and these proteins were utilized to demonstrate that Biacore could be used to determine the kinetics and stoichiometry of the interaction (61). Traditionally, this biosensor technology has also been utilized to investigate poxvirus-host PPIs. For example, Nuara et al. made mutations in the ECTV-encoded IFN-γ binding protein and utilized SPR to identify the specific interactions needed for binding to IFN-γ (118). A comprehensive review of SPR technology and its use for investigating specific poxvirus proteins that are involved in modulating the host immune response was described by Seet et al. (143). Newly developed high-throughput biosensor technologies for PPI, including Octet, have been compared to SPR (Biacore), which was the first technology commercially available and has been considered the "gold standard" (135) for evaluating individual PPIs.

CONCLUDING REMARKS

Poxvirus proteomic studies have provided valuable information and generated new insights into viral pathogenesis and host-virus interaction dynamics. Undoubtedly, these studies will also have an impact on the development of novel therapies and safer vaccines. High-throughput Y2H approaches and analyses of protein complexes by using affinity tag purification and coimmunoprecipitations have yielded valuable PPI maps. In addition, advances in MS methods coupled with the development of proteomic approaches have greatly facilitated the detection and identification of even low-abundance binding partners. Coupled with the rapid increase in the number of high-resolution structures being reported for poxvirus proteins, the data sets from these proteomic studies promise to substantially expand our knowledge of viral interactions with the host and how these viruses are able to evade the host immune responses. However, it is important to stress that information from proteomic studies should always be considered just the start of an investigation into host-pathogen dynamics rather than an end point, and validation of these results and evaluation of their functional in vivo significance should follow.

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Kim Van Vliet received an M.S. degree in biological sciences in 2002 from Florida Atlantic University, where she trained in the laboratory of Michael Roner studying the reovirus nonstructural proteins μNS and σNS. In 2007, she received a Ph.D. degree from the University of Florida, where she studied adeno-associated virus capsid assembly and packaging in the laboratory of Richard O. Snyder. She then joined the laboratory of Grant McFadden at the University of Florida and began studies of cowpox virus-host protein interactions to identify viral proteins that may be suitable for development as therapeutic agents.

Mohamed R. Mohamed received his Ph.D. from the State University of New York (SUNY) at Buffalo, Buffalo, NY, in 2001. After initial postdoctoral studies at SUNY, he joined the University of Florida in 2007. His research focuses on how poxviruses interfere with and manipulate the diverse host immune responses, in particular the NF-κB pathway. He is currently holding an associate professor position in the Department of Biochemistry, Ain Shams University, Cairo, Egypt.

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Leiliang Zhang pursued graduate studies at Peking University, Beijing, China, where he obtained his Ph.D. degree in biochemistry and molecular biology in 2004. After initial postdoctoral studies at Brigham and Women's Hospital, Harvard Medical School, Boston, MA, he joined the McFadden laboratory in 2007. He is interested in understanding the mechanism of how host factors participate in viral pathogenesis.

Nancy Y. Villa received her Ph.D. in biochemistry from the University of Florida in Gainesville, FL, in 2007. She then joined Grant McFadden's lab to work as a postdoctoral associate in virology. Her research is focused on studies of virus entry mechanisms for two different poxviruses using cancer cells as model systems. She is also interested in viral proteomics and has validated vaccinia virus-host protein interactions originally identified by yeast two-hybrid screening.

Steven J. Werden is currently completing his Ph.D. from the University of Florida. His research focuses on understanding the elaborate mechanisms by which poxvirus-encoded proteins micromanipulate the signal transduction pathways of infected cells to promote an intracellular environment for virus replication.

Jia Liu received her Ph.D. from the University of Florida College of Medicine in December 2006 for studies of infectious disease caused by herpes simplex virus and gene therapy of ocular herpes simplex virus infection. In 2007 she started postdoctoral training in poxvirus pathogenesis, poxvirus-host interaction, and oncolytic cancer therapy using poxviruses.

Grant McFadden received his Ph.D. from McGill University in Montreal, Canada, in 1975. He held several academic positions in Canada until 2006, when he relocated to the University of Florida. His scientific expertise is on how viral pathogens interact with the host immune system. His laboratory studies the mechanisms that determine the tropism and host range of poxviruses and the molecular basis of how certain poxviruses can sometimes cause zoonotic infections in humans.